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We have prepared manganic porphyrins, as mimics of SOD activity. Several generalizations follow:

a. *In vivo* action may involve cycles of reduction by NAD(P)H, or GSH, and reoxidation by O_2^- : whereas the *in vitro* cycle involves O_2^- in both reduction and oxidation steps.

b. Action of mimics on bacterial cells is only a rough indicator of their action on mammalian cells; because of differences in uptake of the compounds.

c. Redox potential is more important than net charge, for activity in the *in vitro* assay. The most active compound we have prepared to date is the Mn(III) 2,3,7,8,12, 13,17,18-octabromo-5,10,15,20-tetrakis (N-methyl pyridinium-4-yl)porphyrin. Since the electron withdrawing effect of Br<Cl<F, we will also explore the synthesis and properties of the octachloro and octafluoro analogues. Bromination increased the rate constant by ~50-fold. Chlorination or fluorination might provide even greater improvements.

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FOREWORD

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Marvin Friedrich 5/16/96

PI - Signature Date

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Introduction

Nature of the Problem and Background

It has been established that O_2^- is commonly generated in biologial systems and that it influences both physiological and pathological processes. The relevant literature has been reviewed (1-13). Since superoxide dismutase (SOD) protects against the deleterious actions of O_2^- in numerous pathologies, it has long been apparent that compounds which could mimic the activity of SOD would exert useful effects in ameliorating oxygen toxicity, oxygen-dependent toxicities, inflammations, reperfusion injuries, hemorrhagic shock, burn shock, sepsis, hypertension, and other conditions as well. A massive literature reporting the results of this search for an SOD-mimic has developed. A few recent reports can serve as an entree to this literature (14-17). To date no mimic has been found which equals the activity, stability, and specificity of the native SODs.

Our recent work with Mn(III) porphyrins, bearing substituents on the methine bridge carbons, opens the door to new opportunities in this search for SOD mimics. In brief, we have compounds whose activity in catalyzing this dismutation is represented by a second order rate constant of $4 \times 10^7\ M^{-1}s^{-1}$. This is only 1% as great as the catalytic activity of SOD, yet these compounds provided protection *in vivo* which approached that provided by SOD itself. This apparent paradox is due to the circumstance that the two rate constants for the catalytic cycle of the Mn(III) porphyrin are very different. Thus:

$$O_2^- + \text{Mn(III) porphyrin} \xrightarrow{k_1} O_2 + \text{Mn(II) porphyrin} \qquad 1)$$

$$k_1 = 2 \times 10^7\ M^{-1}\ s^{-1}$$

$$O_2^- + \text{Mn(II) porphyrin} + 2H^+ \xrightarrow{k_2} H_2O_2 + \text{Mn(III) porphyrin} \qquad 2)$$

$$k_2 = 4 \times 10^9\ M^{-1}s^{-1}$$

Further, the Mn(III) porphyrin is rapidly reduced within the cells at the expense of NADPH and of GSH, and possibly at the expense of other cellular reductants, as well. This has the consequence that the Mn(III) porphyrin acts *in vivo* not as a catalyst of the dismutation of O_2^-, but rather as an NADPH/GSH:O_2^- oxidoreductase. When it acts as an SOD-mimic *in vitro* its activity is limited by the slow step of the catalytic cycle, whereas *in vivo* its rate of scavenging of O_2^- reflects the rate of the very fast oxidation of the

Mn(II) porphyrin by O_2^- . It is thus 100 times more active, as a scavenger of O_2^- *in vivo* than we would have expected from its SOD-mimetic activity measured *in vitro*.

Purpose and Methods

The SODs were selected, during evolution, to catalyze the dismutation of O_2^- . They are nearly perfect in that the rate constants for both steps of the catalytic cycle are close to diffusion-controlled.

SOD-mimics, in contrast, may exhibit very different rate constants for the two half reactions of the catalytic cycle. In the case of the Mn(III) porphyrins, this difference is two orders of magnitude. In the case of other potential mimics the difference may be greater, such that the activity in the standard *in vitro* assays, which is always rate-limited by the slowest step, may be judged trivial. Compounds which may be very useful scavengers of O_2^- *in vivo* will thus fail to be detected in the assays designed for measuring the rate of dismutation of O_2^- . We propose that assays designed to measure the fast step of the catalytic cycle will better predict *in vivo* efficacy and we will devise such assays. Compounds, such as the Mn(III) porphyrins will then be tested for their abilities to protect ceils against the toxicity of oxygen and against the oxygen-dependent toxicities of redox active compounds such as viologens, quinones, dyes, aryl nitro compounds, and benzofurazans. Compounds which provide such protection without exhibiting too much toxicity will then be tested for their ability to ameliorate inflammations, reperfusion injuries, hemorrhage shock, burn shock, hypertension, and other free-radical-related pathologies. We expect that pharmaceutically useful compounds can be found and tested in this way.

Additional considerations pertinent to the efficacy of SOD-mimetics are redox potential of the catalytic center and the net charge of that center. The active sites of all known superoxide dismutases have standard redox potentials of close to +300 mV. That is ideal because it is midway between the O_2^-/O_2 potential and the H_2O_2/O_2 potential. The redox potential of the manganic porphyrins can be raised by adding electron withdrawing substituents such as -Br, -Cl, -F, or -NO_2. A more positive redox potential, approaching +300 mV, will make the rate constants for the two halves of the catalytic cycle more nearly equal. This in turn will allow the mimetics to act as true catalysts of the dismutation of O_2^-, both *in vitro* and *in vivo*.

The SOD enzymes also provide for electrostatic facilitation which speeds the catalytic process by guiding the O_2^- to the active center. We wish to find out how important that is in the design of mimetics.

Methods

Various Mn-porphyrins are being made and tested for activity in the xanthine oxidase/cytochrome *c* assay for SOD activity and for ability to protect *E. coli*, and human umbilical vascular endothelial (HUVEC) cells, against oxygen toxicity and against paraquat toxicity. Since it would also be useful to achieve a catalase mimetic, we are also testing for catalase activity.

The octabromo TMPyP was prepared as described by R. A. Richards et al. (1996) Inorg. Chem. 35, 1940-1944. The preparations of the other compounds used are similarly taken from the literature. Cyclic voltammetry has been done on several of the manganic porphyrins and the only one with a positive $E^{\circ'}$ was the manganic octabromo TMPyP ($E^{\circ'}$ = +410 mV). Thus, for comparison, $E^{\circ'}$ for Mn(III)TMPyP was -200 mV and it was ~100 fold less active than the octabromo compound. We are clearly on the right track in seeking to approach the ideal $E^{\circ'}$= +320 mV. We will assess the importance of electrostatic guidance by preparing the β-octabromophenyl sulfonato-substituted porphyrin. It should have $E^{\circ'}$ close to +400 mV, but with a net negative charge instead of a net positive charge. We already have the β-octabromo benzoato compound but have not yet assayed it for SOD activity. However, at 10 μM, it virtually completely protects L2 cells against kill by f.met.leu.phe-activated neutrophils (see attached figure). Using β-halogenation to increase $E^{\circ'}$ of the manganic porphyrins is clearly a promising route to useful mimics of SOD.

Body

Several dozen compounds have been prepared thus far (see Appendix for structures and identifying numbers). All of these compounds have been assayed for SOD and catalase activities and some have further been tested for abilities to protect *E. coli* and HUVEC cells (see data provided in index).

Most recently we have prepared and begun to examine the octabromo compound (structure in Appendix). This is water soluble and very active in the SOD assay. It will be assayed for catalase activity and will be put through our cell culture screen. It is the most active SOD mimetic seen to date and it, or the chloro or fluoro analogues, will hopefully be candidates for in vivo testing.

I. Objectives - To produce and test synthetic compounds which catalyze the dismutation of O_2^- into H_2O_2 plus O_2, as does superoxide dismutase (SOD). The desired SOD-mimic should approach the catalytic activity of the native SOD; should be stable to chelating agents such as ADP, ATP, citrate and even EDTA; should be capable of crossing cell membranes; and should be non-toxic. Such a compound could be useful in treatment of inflammatory diseases,

reperfusion injuries, autoimmune diseases and even senescence. We are exploring metallo porphyrins because they are very stable, leave the metal center accessible to solvent, and can be modified both with respect to redox potential and charge.. We have focussed on manganic porphyrins because Mn(II), unlike Fe(II) or Cu(II), does not participate in Fenton chemistry and is thus less likely to cause toxicity.

We have two guiding principles: <u>One</u> is redox potential. An ideal redox potential for a catalyst of the dismutation of O_2^- should be ~+300 mV. That places it midway between the redox potentials of the O_2^-/O_2 couple and the H_2O_2/O_2^- couple. This allows the two half reactions of the catalytic cycle vis:

a. $Me^n + O_2^- \rightleftharpoons Me^{n-1} + O_2$

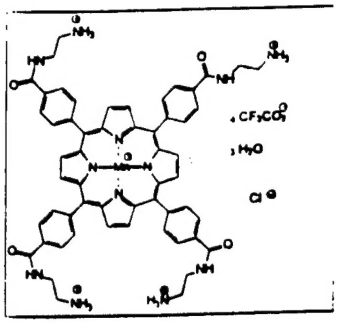
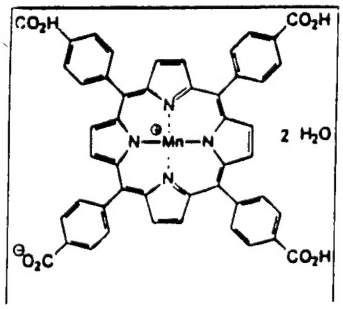
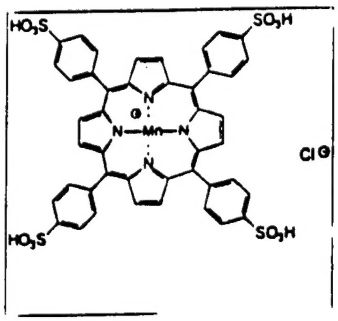
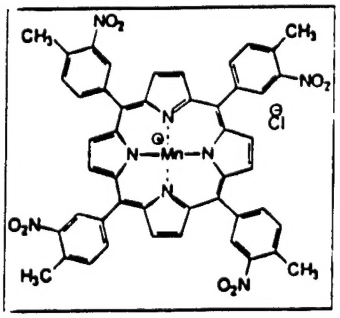
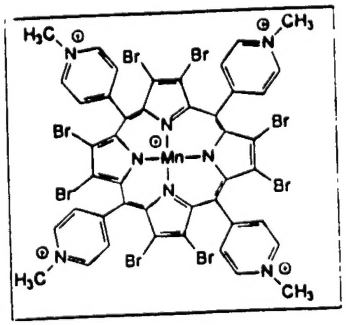
b. $Me^{n-1} + O_2^- + 2H^+ \rightleftharpoons Me^n + H_2O_2$

to proceed with approximately equal free energy decreases. In fact the redox potentials of all the SODs are close to +300 mV (18-21) even though they have different metals at the active center. Clearly the ligand fields have been modified by evolution to give optimal catalytic efficiency.

Our <u>second</u> guiding principle is electrostatic guidance. We expect that a polycationic compound will attract the anionic O_2^- and thus facilitate the catalytic process. The operation of the native SODs have been shown to occur with the aid of electrostatic facilitation (22-28). It thus appears that this is enough of an advantage to have been selected for over the long reaches of evolutionary time.

II. <u>Milestones</u> - We have prepared and partially evaluated a number of manganic porphyrins in order to test our guiding principles. See structures on the following page. Note that the compound with the greatest SOD activity (#5) was an octabromo porphyrin with N-methyl pyridyl substituents on the methine bridge carbons. Indeed on a per mg basis it is more active than native SOD. On a molar basis it has 1/10 the turnover rate of native SOD. This octabromo compound was examined by cyclic voltammetry and its E_0 was found to be +400 mV; undoubtedly due to the electron withdrawing effect of the β bromines. We are very pleased with this octabromo manganic porphyrin. It can be assayed in the presence of excess EDTA and it protects *E. coli* and human umbilical vein endothelial cells (HUVEC) against the toxicity of paraquat. It provides the electrostatic facilitation due to its halo of cationic N-methyl pyridyl groups and it has the optimal redox potential. It increases our confidence in the guiding principles stated at the outset.

III. <u>Achievements</u> - We have become more familiar with porphyrin chemistry and thus are now confident that we can prepare the compounds we want to test. We have prepared a series of manganic porphyrins, both anionic and

#	Structure	Activity Units/mg
1		11.9
2		0.0
3		1.1
4		55.6
5		8,770.0

cationic, and with a range of redox potential. Our best compound, i.e. the octabromo-N-methylpyridyl compound (#5), is amazingly active as a SOD mimic with a rate constant for reacting with O_2^- of 2 x 10^8 $M^{-1}s^{-1}$. Furthermore it protects both bacterial and mammalian cells against the toxicity of paraquat and this toxicity is largely due to the generation of O_2^- by the redox cycling of the paraquat.

IV. Conclusions - Very active, stable, and water soluble mimics of SOD activity can be based on manganic porphyrins. Compound 5 is good enough to go into animal trials. New compounds in which the electron withdrawing effect is provided by chloro, fluoro, or nitro groups should be made. It may be possible to exceed the activity of the octabromo compound. In any case it is desirable to have a selection of very active compounds so that the least toxic of them can proceed to clinical trials.

V. Expected Achievements for Next Year - We shall need to prepare gram quantities of compound #5 so that enough will be available for testing in mice. This will be done. At the same time we will try to prepare analogues of compound #5 in which the bromine atoms are replaced by chlorines, fluorines, or nitro groups. Since the electron withdrawing effects of these substituents exceed that of bromine it may be possible to achieve optimal activity with a smaller number of groups. Thus a tetrachloro or tetranitro compound may have optimal activity. We anticipate making and testing such compounds.

VI. Methods - The methods for preparing and testing some of the manganic porphyrins have been described in our previous publications (29-32). The octabromo compound (#5) was prepared as described by Richards et al. (33) who made the ligand, but did not insert Mn into it. We found the metallation to be facile. An octabromo sulfonatophenyl porphyrin has been described (34) and we plan to make its manganic complex for testing.

Conclusions

We are encouraged by the properties of the octabromo compound. its catalytic rate constant is within an order of magnitude of that exhibited by the SODs themselves. Further it may be possible to gain further advantage by substituting with more electronegative halogens such as chlorine or fluorine. We are also encouraged by the abilities of the much less active SOD mimics, which have already been shown to protect bacteria and HUVEC cells against oxygen toxicity and against the oxygen-dependent toxicities of paraquat. We thus can expect even more dramatic protections by the octahalo compounds. When that has been demonstrated we will be ready to undertake their evaluation in animals.

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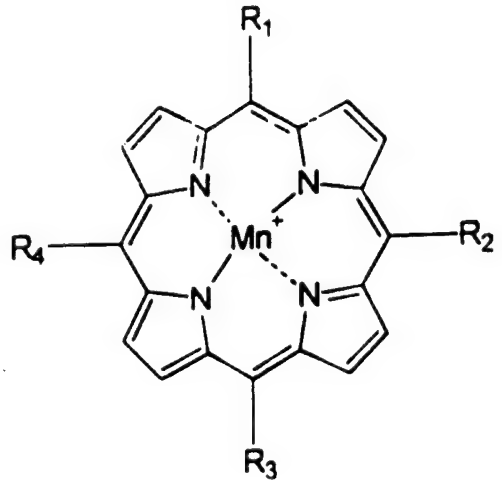
Appendix

Sructure Activity Relationship of Acid/Ester Derivatives of Manganic Meso-porphyrins



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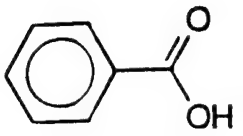
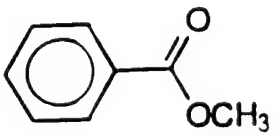
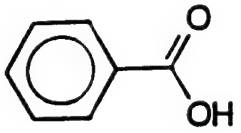
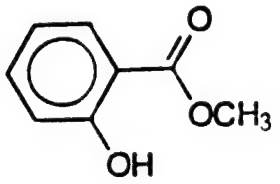
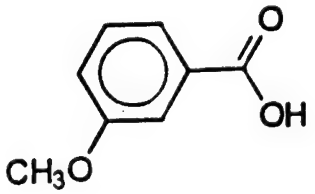
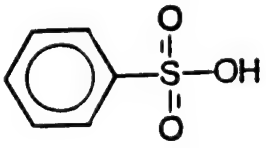
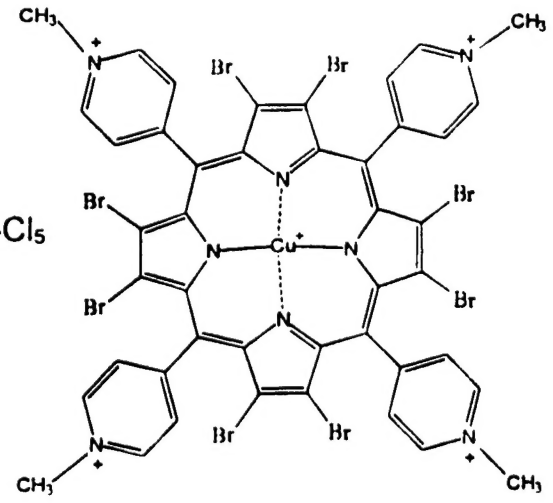
R_{1-4}		30
R_{1-4}		5
R_1		25
R_{2-4}		
R_{1-4}		50
R_{1-4}		20

Structure of Compounds

Compound Name:	Cu(II) OBTMPyP
Compound Code:	AEOL-11101
Lot Number:	IH-1
Molecular Weight:	1537
Molecular Formula:	$C_{44}H_{16}N_8Br_8Cu\text{-}Cl_5$
Quantity:	
Melting Point:	
TLC Analysis:	
UV-Vis Analysis:	Attached, λ_{max} = 457 nm: Em = 7.41 x 10^4 M^{-1} cm^{-1}
HPLC Analysis:	
Solubility:	water soluble
Notes:	

Structure:



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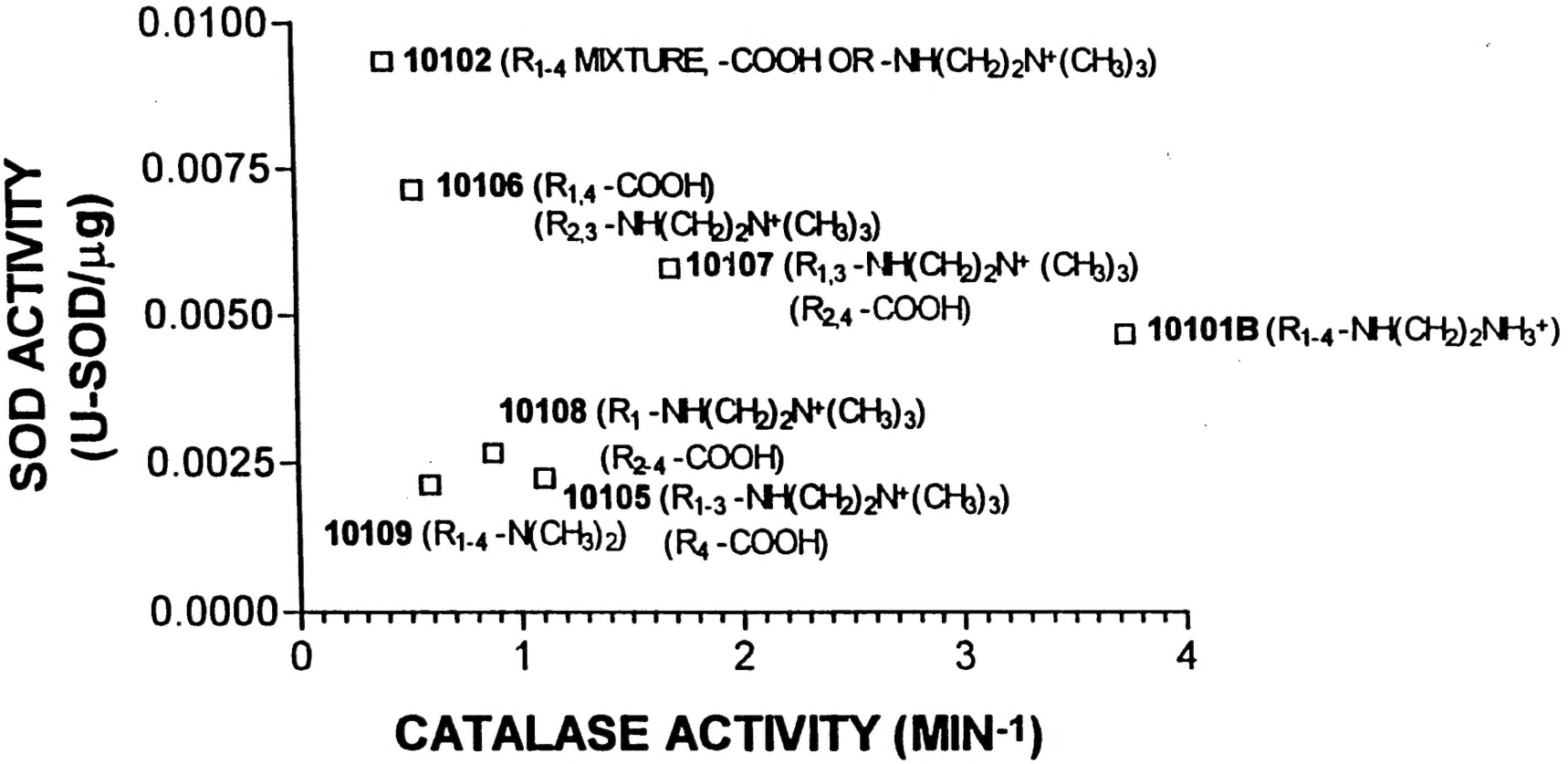

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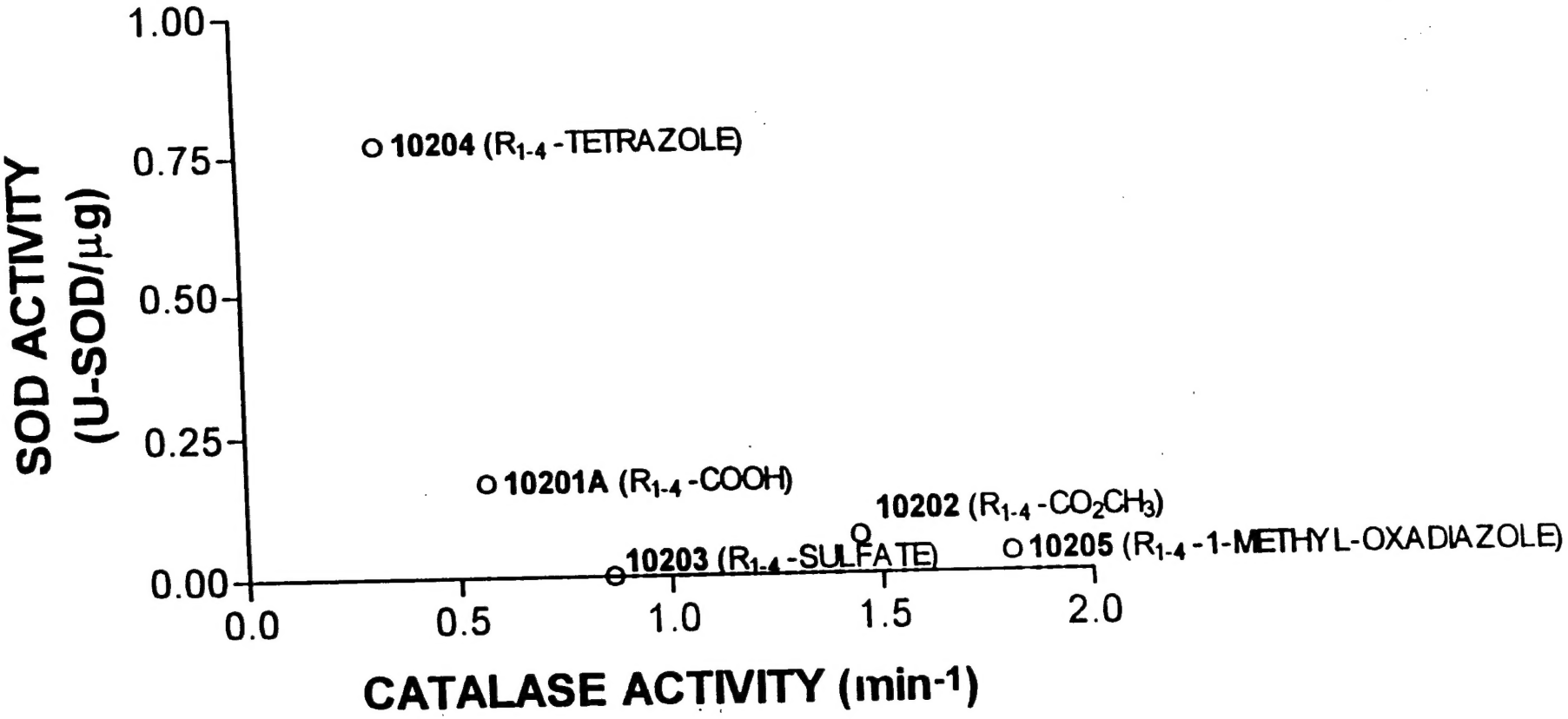

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